CONTRACT NUMBER: N62645-12-C-4055

TITLE: Synthetic platelets: Intravenous infusible nanoparticles to promote hemostasis and survival following liver injury in swine

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REPORT DATE: August 12, 2014

TYPE OF REPORT: Final

PREPARED FOR: Advanced Medical Development
Naval Medical Research Center
503 Robert Grant Avenue
Silver Spring, MD 20910

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
08-08-2014	Final	13 Aug 2012 - 12 Aug 2014

4. TITLE AND SUBTITLE

Synthetic Platelets: Intravenous infusible nanoparticles to promote hemostasis and survival following liver injury in swine

5a. CONTRACT NUMBER
N62645-12-C-4055

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Erin Lavik, ScD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Advanced Medical Development
Naval Medical Research Center
503 Robert Grant Avenue
Silver Spring, MD 20910-7500

10. SPONSOR/MONITOR'S ACRONYM(S)

AMD NMRC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

DISTRIBUTION A. Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Explosions account for 79% of combat related injuries and involve multiple organs and internal bleeding. Pressure dressings and absorbent materials (e.g. Quik-clot®) are effective but are limited to compressible and exposed wounds. There is a need for treatment for internal bleeding.

Synthetic platelets were developed based on functionalized nanoparticles that halve bleeding in a rat major femoral artery injury following intravenous administration. The synthetic platelets are safe and stable at room temperature. They halt bleeding significantly faster than other treatments including recombinant factor VIIa.

This proposal tested these synthetic platelets in a large animal model of trauma that mimics human trauma. A swine liver injury model was used to determine the safety, efficacy and dosing of the synthetic platelets. This model was developed to mimic non-compressible injuries sustained by military personnel and permits direct comparison to other hemostatic interventional studies. It was found that by carefully engineering the particles, one could stop bleeding without triggering complications.

15. SUBJECT TERMS

blood loss, hemostasis, internal bleeding, nanomedicine

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			AMD Program Manager, NMRC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)* 301-319-7408

N62645-12-C4055 Final Report. CASE WESTERN RESERVE UNIVERSITY (RES122947)

Advanced Medical Development Program FY2012/2013

Proposal Number:	N62645-12-C-4055	**P.I. Name:**	Erin Lavik, ScD
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		P.I. Organization:	Case Western Reserve University
Proposal Title:	**Synthetic platelets: Intravenous infusible nanoparticles to promote hemostasis and survival following liver injury in swine**		

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Introduction

Explosions account for 79% of combat related injuries (Ramasamy et al. 2008) and involve multiple organs and internal bleeding (Warden 2006). Pressure dressings and absorbent materials (e.g. Quik-clot®) are effective bu are limited to compressible and exposed wounds. Therefore, a treatment for internal bleeding is needed Synthetic platelets based on functionalized nanoparticles have been developed, which halve bleeding in a ra major femoral artery injury following intravenous administration. The synthetic platelets are safe and stable a room temperature. They halt bleeding significantly faster than other treatments including recombinant facto VIIa. The pivotal work at the core of this contract was the development of synthetic platelets for the porcine model as a critical step towards translating the technology to humans and the performance of dosing studies to determine the effective dose for these particles in the swine.

Body

The following set of tasks was addressed within this statement of work:

- Fabrication and characterization of synthetic platelets
- Dosing study
- Determination of degree of efficacy in halting bleeding based on optimal dosing
- Biodistribution study
- Histological assessment of organs
- Thromboelastography (TEG) analysis of blood samples

The following were the deliverables:

- Determination of impact of synthetic platelet administration on bleeding and survival following blunt trauma
- Determination of safety of synthetic platelet administration on survival following blunt trauma
- Determination of dependence of synthetic platelet biodistribution on dosing
- Determination of any nonspecific clot formation
- Determination of safety
- Determination of the integrity of the clots formed with and without synthetic platelets

This report summarizes results from the tasks and deliverables. It also includes major, unexpected findings regarding the characteristics of intravenously administered nanoparticles that trigger complement activation related pseudoallergy (CARPA) (Szebeni et al. 2012). This finding necessitated the re-engineering of the hemostatic nanoparticles from the molecule to the therapy, which has led to a new understanding of the

nanomaterials characteristics that trigger complement activation. This has broad implications for the field of nanomedicine and has led to a patent, PCT/US2014/034176.

Fabricating and characterizing synthetic platelets: Generation 1 particles

The initial task was scaling up the fabrication of the synthetic platelets (GRGDS-based nanoparticles) and the control nanoparticles.

This was accomplished, and the particles characterized using nuclear magnetic resonance (NMR), scanning electron microscopy (SEM), zeta sizing, and peptide analysis. Representative data for the particles is in Figure 1 below. The nanoparticles and controls were consistent with all of the previously-developed nanoparticles.

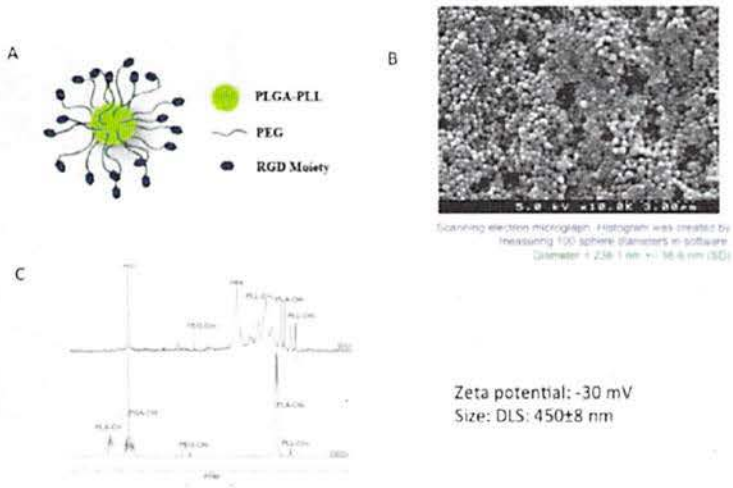

Figure 1: Synthetic Platelets. (A) Schematic of synthetic platelets showing PLGA core, PEG arms, and RGD-based peptide for binding with activated platelets. (B) SEM micrograph of particles. Based on the SEM, the particles are approximately 250 nm. DLS measurements confirm the hydrodynamic diameter of the particles to be closer to 450 nm and the zeta potential to be -30 mV. The NME confirms the chemistry and the presence of the PEG arms on the outside of the particles. This data is identical to the results found in the researchers' previous work (Shoffstall et al. 2013).

Dosing study: Generation 1 particles

It is known from previous work that these particles are effective in stopping bleeding at a dose of approximately 30 mg/kg in the rat (Shoffstall et al. 2013). Based on this knowledge, test dosing from 1 mg/kg to 100 mg/kg in swine was decided on to see if a dose could be determined that replicated the reduction in blood loss and increase in survival seen in the rodent liver injury model (Shoffstall et al. 2013).

Figure 2 shows the development of the model with saline administration. The swine that received saline survived for the entire 240 minutes with an average blood loss of approximately 800 ml.

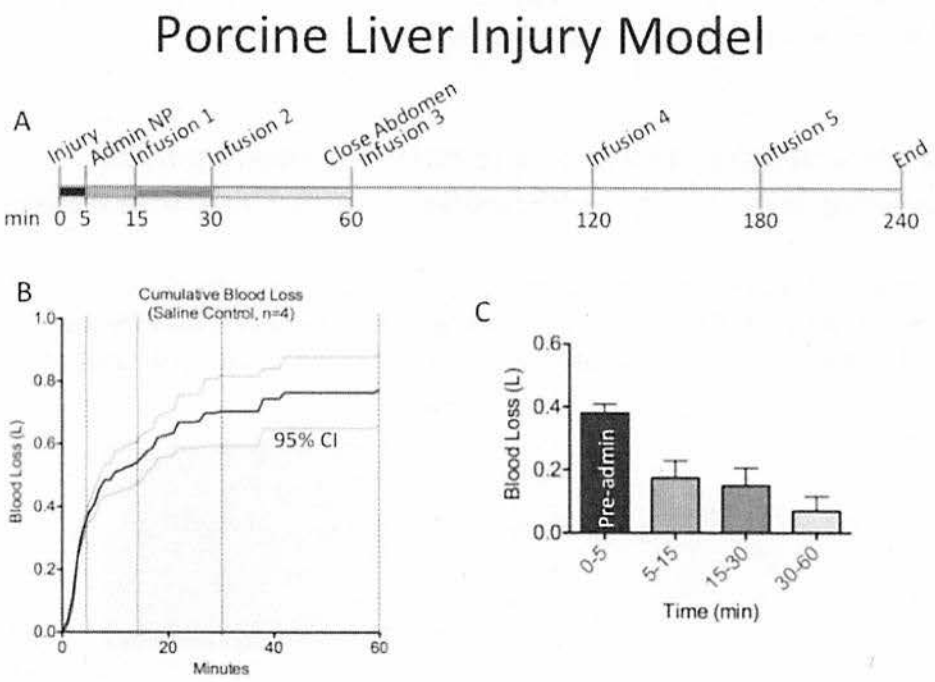

Figure 2: Development and validation of the porcine model with saline administration. The treatment (saline in this case) is administered at 5 minutes post injury (n=3).

Dosing began with a 2 mg/kg dose in the swine. The swine bled out in 9 minutes. The control nanoparticle group bled out in 8 minutes. This was definitely an unexpected response. The preliminary hypothesis was that the dose was too high. It is possible, with a high enough dose, to saturate all of the activated platelets and exacerbate bleeding. Therefore, a dose of 0.2 mg/kg was attempted. The swine bled out in 7 minutes. The dose was halved and 0.1 mg/kg tested. The data is in Figure 3.

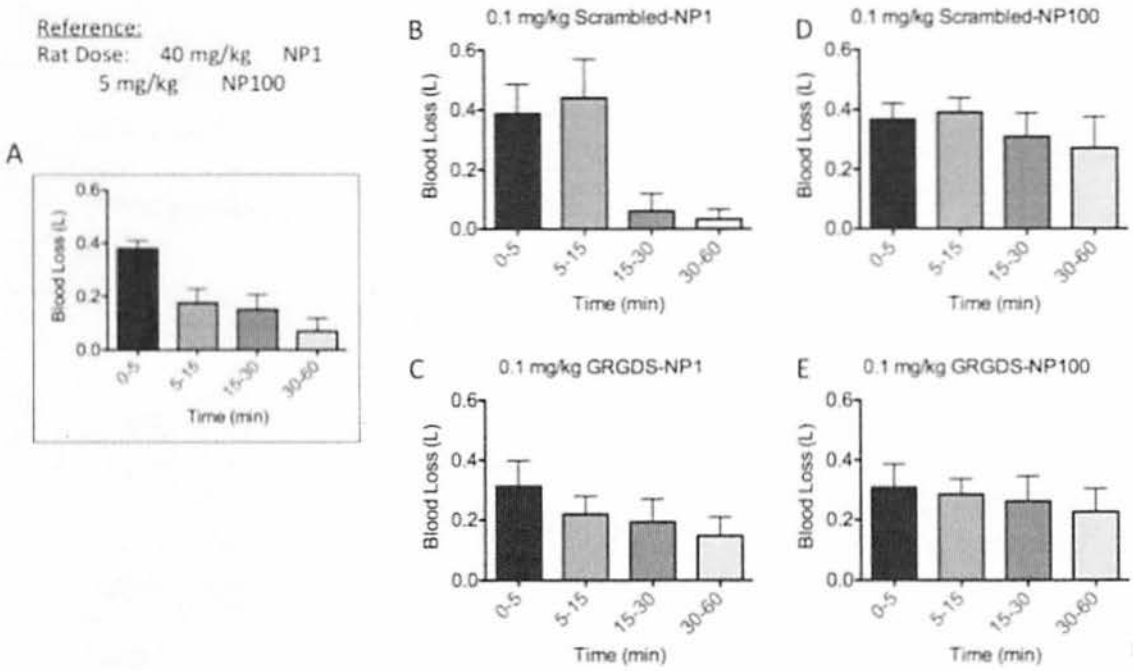

Figure 3: (A) Saline data for comparison. (B) Control nanoparticles with low peptide density. (C) 0.1 mg/kg dose of synthetic platelets that was effective in rats at 40 mg/kg. Some exaggeration of bleeding, even at this low dose is present. (D) Control nanoparticles with high peptide density (effective in rats at 5 mg/kg). Some exaggeration of bleeding with the control nanoparticles is seen. (E) High density peptide synthetic platelets at 0.1 mg/kg also exaggerate bleeding. N=3 for all data.

Even at the lowest dose, 0.1 mg/kg, all of the groups, controls, and active synthetic platelets at both the low and high density peptide formulations exacerbate bleeding. This suggests the particles are not directly affecting the platelet plug, but are affecting something else. Many of the swine were observed to flush immediately following administration of any of the particles regardless of peptide. Based on this response, the particles were injected in a naïve swine to see if the particles, alone, were having some effect.

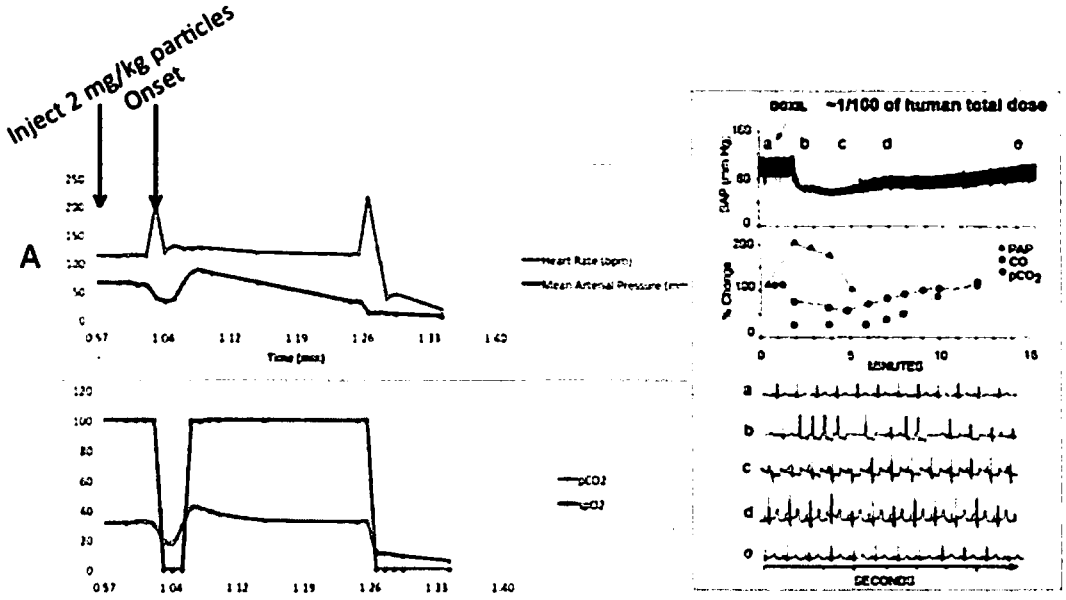

Figure 4: Administration of 2 mg/kg of synthetic platelets in a naïve, uninjured swine. (A) Within a minute of the intravenous administration of the synthetic platelets, the heart rate spiked, the gas levels plummeted, and the mean arterial pressure dropped then spiked. The response was reproducible with more nanoparticles. (B) An investigation of the literature revealed that a similar response had been seen before in swine by Szebeni et al. (Szebeni et al. 2012).

Even without the confounding variable of an injury, the administration of nanoparticles was inducing a strong physiological response within one minute of the infusion. Based on this information, it was determined from the literature that there was a strong probability that the nanoparticles were triggering complement activation related pseudoallergy (CARPA) in both swine and humans (Figure 5) (Johnson et al. 2011; Szebeni et al. 2012; Szebeni et al. 2012). CARPA is a significant issue in many nanomedicines.

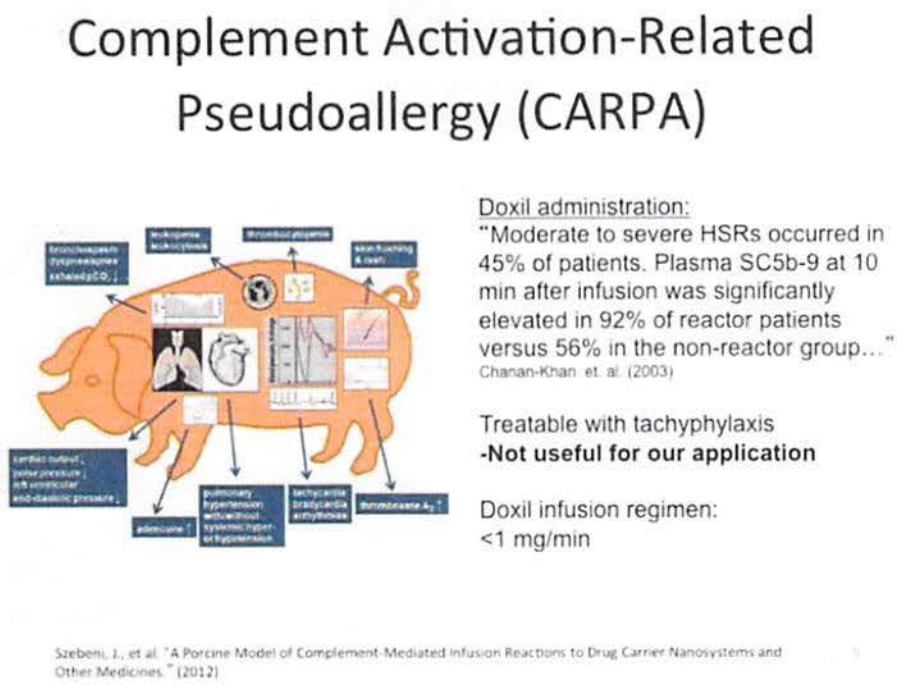

Figure 5: Schematic explaining CARPA response as well as the relationship to current therapies. CARPA is seen with most of the major nanomedicines. Slow infusion can mitigate it to a degree, but that is not possible for the technology in question.

In fact, Doxil, the PEGylated liposomal formulation of doxorubicin, triggers mild to severe cardiopulmonary responses in patients that disappear over several infusions in a process of self-induced tolerance called tachyphlaxis (Szebeni et al. 2012). The solution, traditionally, is to administer the liposomes at extremely slow rates (1 mg/min) along with drugs to treat the cardiopulmonary responses (Goram and Richmond 2001).

However, a slow infusion is not possible for a hemostatic agent because the patient could bleed out before the particles arrive. Therefore, it was necessary to engineer the synthetic platelets to stop bleeding without triggering CARPA. Szebeni's work suggests that charge may modulate the response, so the effect was explored of the zeta potential on the nanoparticles on the CARPA response.

Fabricating and characterizing synthetic platelets: Generation 2 particles

Nanoparticles with positive, negative, and neutral zeta potentials were developed and administered to naïve, uninjured swine at doses that have been observed to reliably trigger the CARPA response. Particles with neutral zeta potentials did not lead to the rapid change in O2, heart rate, or blood pressure that had been seen previously. The acceptable "neutral" zeta potential range was narrowed down to greater than -3 mV but less than 3 mV. Table 1 summarizes these findings.

Table 1: Zeta potential on nanoparticles mediates CARPA response

Polymer Nanoparticle	Dose (mg/kg)	Zeta Potential (mV)	CARPA?
PLA-PEG	2.0	-30	Yes
PLA-PEG	2.0	-30	Yes
PLGA-PLL-PEG	2.0	-1.3	NO
PLGA-PLL-PEG	2.0	+20	Yes

Now that a clear direction had been found, it had to be determined how to make neutrally charged nanoparticles with the targeting peptide. In all of these tests, nanoparticles without peptides were used.

The RGD-based peptide that has been used for all work up to this point has been a GRGDS peptide, but this leads to a net negative charge on the particles. However, an alternative peptide, cRDG, could lead to neutral particles when coupled (Figure 6).

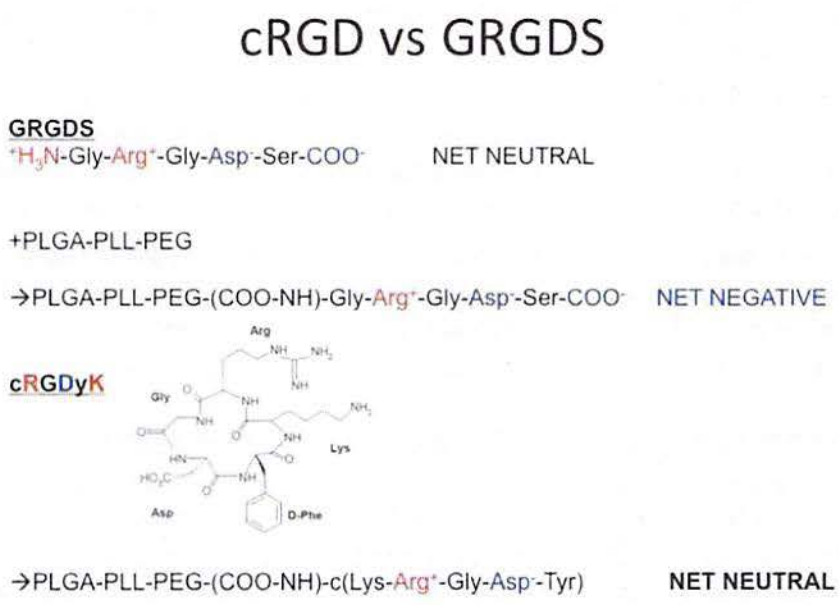

Figure 6: cRGD vs. GRGDS impacts the charge on the nanoparticles.

Neutral cRGD-based synthetic platelets were created. Two things needed to be accomplished before moving to the dosing study: validate that they did not trigger CARPA and test to determine what the effective dose was in rodents as a baseline for the dosing study in swine.

Testing cRGD neutral particles for CARPA effect

Following fabrication of the cRGD-synthetic platelets, it was observed that the neutral zeta potential coupled with the presence of the peptide led to rapid aggregation of the particles. Therefore, the addition of a polymeric excipient was required to avoid particle aggregation and facilitate resuspension of the particles.

cRGD-synthetic platelets with poly(vinyl alcohol) (PVA) were formulated, since it is used extensively in the researchers' clinic, but administration of the PVA-based synthetic platelet formulation triggered CARPA (Table 2). Therefore, the excipients were examined separately and it was determined that while PVA triggers CARPA on its own, poly(acrylic acid) PAA and the poloxamer 188 do not (Table 2).

Table 2: Excipient also mediates CARPA

Polymer Nanoparticle	Excipient	Dose (mg/kg)	Zeta Potential (mV)	CARPA?
PLGA-PLL-PEG	--	2.0	-1.3	NO
Active synthetic platelets (cRGD)	PVA	2.0	-0.79	Yes
--	PVA		--	Yes
--	pAA	1.0	--	NO
--	poloxamer	8.3	--	NO
Active synthetic platelets (cRGD)	poloxamer	2.0	1.12	NO

Based on these findings, testing in the remainder of the work focused on the cRGD-synthetic platelets with poloxamer 188 (Figure 7).

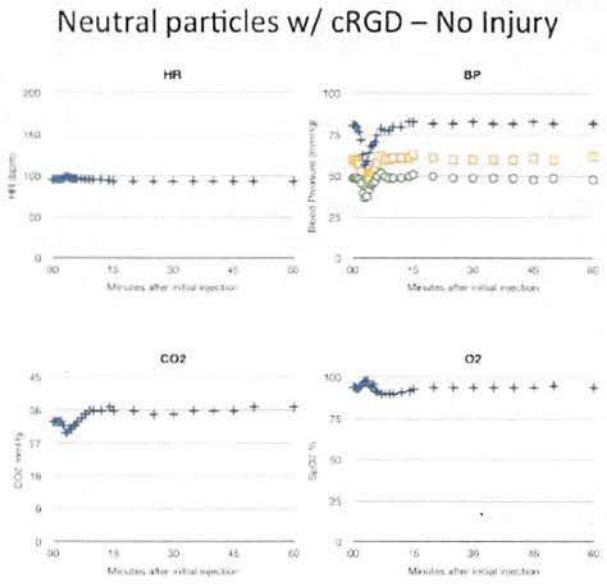

Figure 7: There are no major changes in the physiological parameters associated with CARPA following administration of the cRGD-synthetic platelets with poloxamer 188 in a naïve, uninjured swine.

Testing cRGD neutral particles for hemostatic efficacy in rat model

Now that a formulation that did not trigger CARPA had been identified, it needed to be tested in the rodent model of liver injury to determine the optimal dose for the dosing study in the swine. Because cRGD and GRGDS have different affinities to the glycoprotein IIb/IIIa receptor on activated platelets, the efficacy could be different.

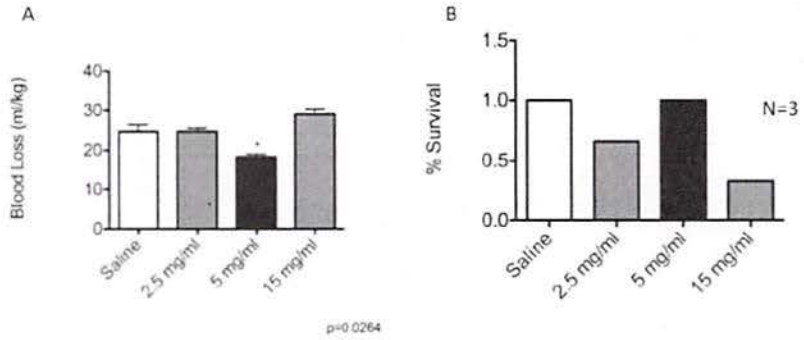

Figure 8: Blood loss and survival with the cRGD-synthetic platelets with poloxamer 188. The 5 mg/ml dose (very similar to the GRGDS particles) was the most effective at reducing bleeding and increasing survival in the small, pilot study.

Dosing study: Generation 2 particles

Now that a baseline had been developed, a dosing study of the cRGD-synthetic platelets with poloxamer 188 in swine was initiated. The doses examined were 25 mg (0.8 mg/kg); 100 mg (3.3 mg/kg); and 200 mg (6.6 mg/kg).

The summary of the dosing study with a n=3 for all groups is in Figure 9. 100 mg (3.3 mg/kg) appears to be the optimal dose with the greatest reduction in bleeding and longest survival time. Power analysis indicates that groups of 6 swine at the optimal dose are needed for statistically significant findings.

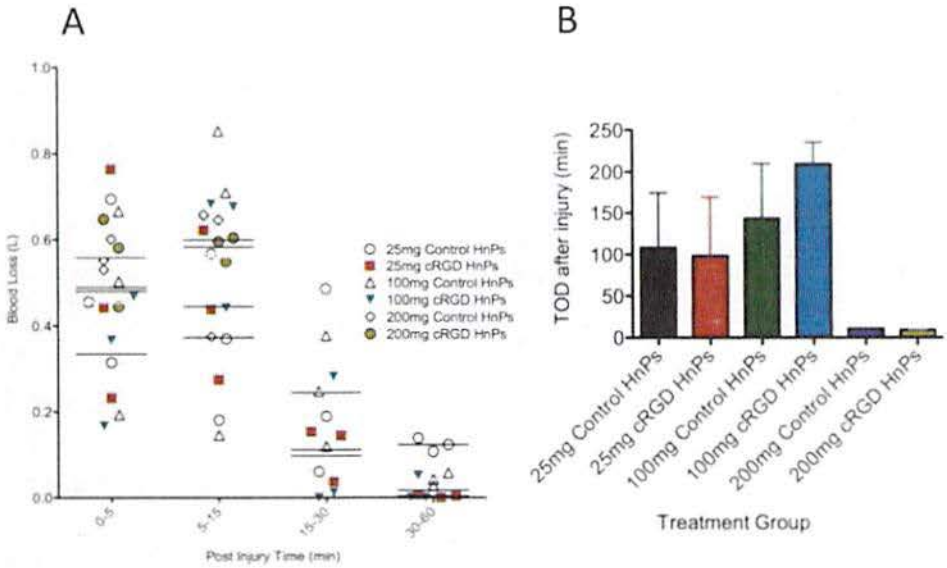

Figure 9: Dosing study for cRGD-synthetic platelets with poloxamer 188 in swine. (A) Blood loss over time for all of the groups. The 100 mg group (3.3 mg/kg) showed the greatest reduction in bleeding post administration as well as the longest survival time in this study (B).

Determination of degree of efficacy in halting bleeding based on optimal dosing

Focus was placed on the 3.3 mg/kg dose, which appears to be optimal for the cRGD-synthetic platelets with poloxamer 188. This dose reduced bleeding at every timepoint post administration and had the best survival time of any of the doses investigated with no signs of CARPA.

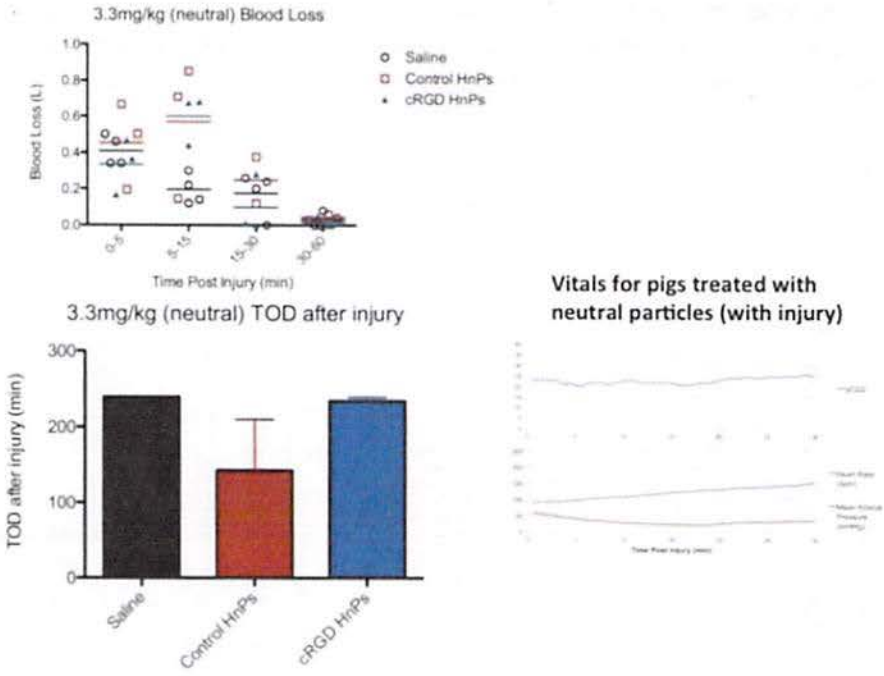

Figure 10: Optimal dose of cRGD-synthetic platelets with poloxamer 188. The formulation reduced bleeding post administration and showed no signs of CARPA.

Biodistribution study

Since CARPA was encountered, coumarin 6 was not included in the formulation because it can be toxic if released from the particles. Therefore, the biodistribution in the swine was not completed.

Histological Assessment of organs

The diameters of all the major vessels that contributed to bleeding in the liver after injury were quantified (Figure 11).

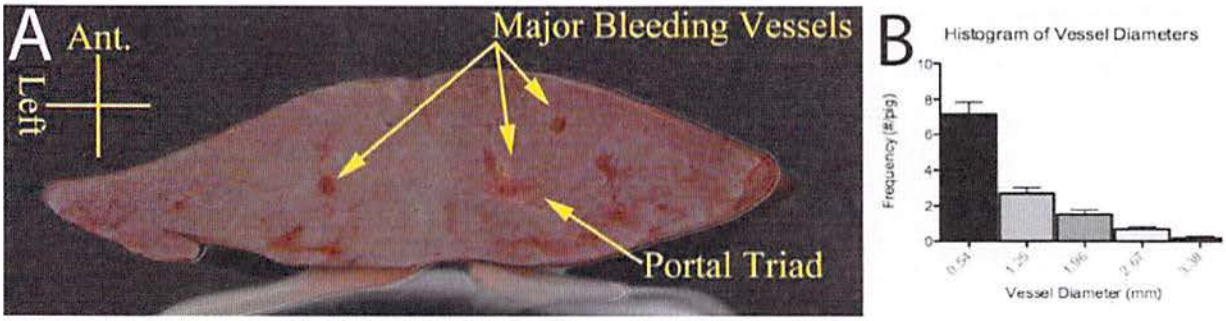

Figure 11: (A) Cross section of a liver following resection. (B) Histogram of the vessel diameters. There were no significant differences in the vessel numbers or diameters between any of the swine used in this study.

Additionally, histology on the livers was performed to look for the structures and any signs of inflammation. No differences were noted in inflammatory cells in any of the sections observed.

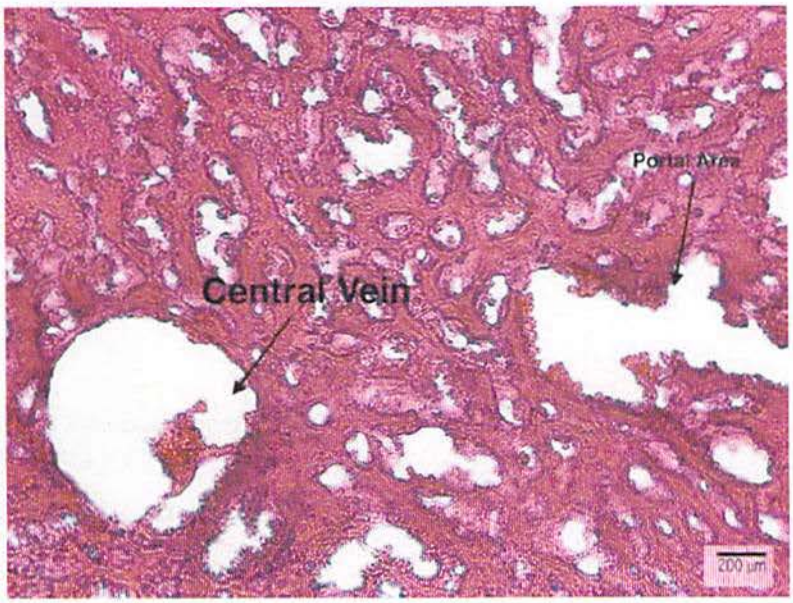

Figure 12: Section of a liver stained with hemotoxylin and eosin.

TEG analysis of blood samples

Rotational thromboelastometry (ROTEM) of the porcine blood samples was performed before and after the injuries and administration of synthetic platelets.

In the CARPA-causing synthetic platelet swine, significant changes were not observed in the clotting time between groups or in the clot firmness (Figure 13). Likewise, significant effects were not seen in the cRGD-synthetic platelets with poloxamer 188. The ROTEM results appeared to be mostly affected by the initial concentration of platelets in the swine (which varied dramatically between animals before injury) and in the time after injury. The particles do not seem to affect the clotting efficacy of the blood.

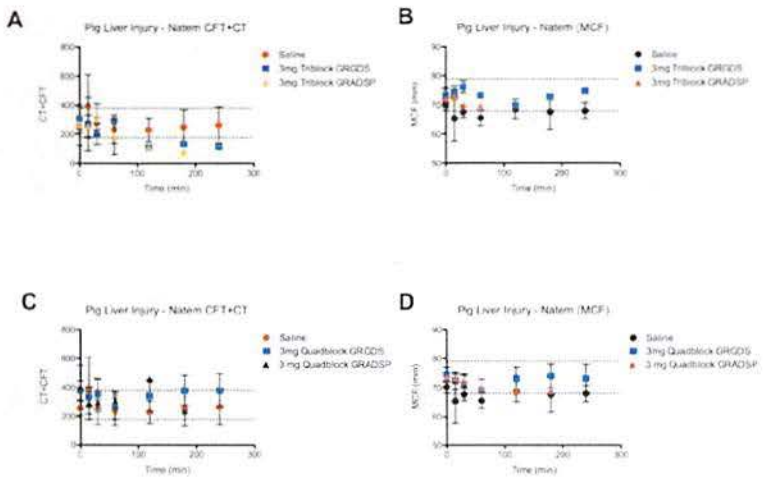

Figure 13: (A) Triblock synthetic platelets with RGD, clotting time. (B) Clot firmness for triblock synthetic platelets. (C) Quadblock synthetic platelets with RGD, clotting time. (D) Clot firmness for the quadblock particles.

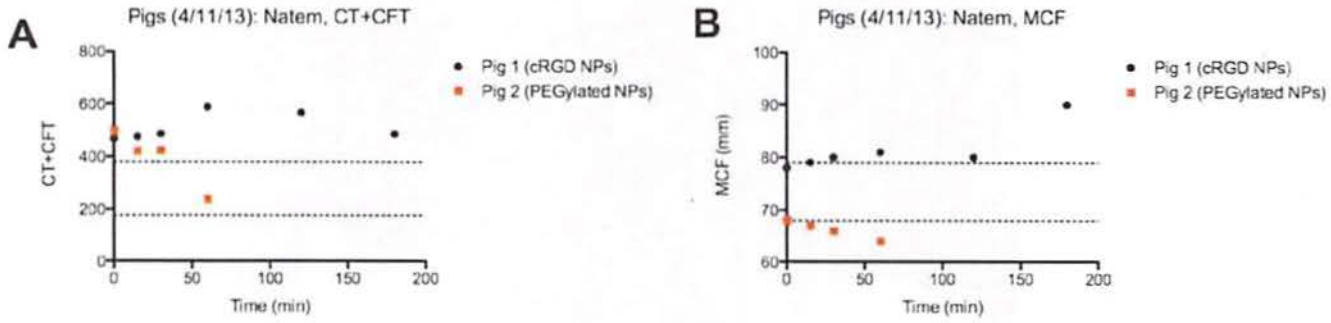

Figure 14: cRGD-synthetic platelets with poloxamer 188 nanoparticle swine. (A) Clotting time. The clotting time does not appear to be different between the treatments. (B) Clot firmness is also not significantly different.

Through all of these tasks, there was the following set of deliverables:

- Determination of impact of synthetic platelet administration on bleeding and survival following blunt trauma
- Determination of safety of synthetic platelet administration on survival following blunt trauma
- Determination of dependence of synthetic platelet biodistribution on dosing
- Determination of any nonspecific clot formation
- Determination of safety
- Determination of the integrity of the clots formed with and without synthetic platelets

Based on the findings, it is believed that the cRGD-synthetic platelets with poloxamer 188 at a dose of 3.3 mg/kg is a safe and effective dose to stop bleeding. Blood samples will continue to be examined for signs of complement changes, but these particles showed none of the manifestations of CARPA. It was also determined that the particles did not appear to affect the integrity of the clots formed based on ROTEM results.

This work lays the foundation for the next generation of hemostatic nanoparticles. However, it is necessary to continue to expand the numbers, look at biodistribution, and determine if higher doses (such as the 200 mg dose) cause any unexpected complications.

Key Research Accomplishments

- Determined that the basic chemistry of nanoparticles can trigger a massive complement response (CARPA)
- Developed a new generation of synthetic platelets (cRGD-synthetic platelets with poloxamer 188) that do not trigger CARPA over a range of doses
- Performed a dosing study in swine following liver injury and determined that 3.3 mg/kg of the new cRGD-synthetic platelets with poloxamer 188 stop bleeding most efficiently

- Determined that the particles do not interfere with clotting, even when the particles trigger CARPA
- Filed a patent on the technology to facilitate commercialization (PCT/US2014/034176)

Reportable Outcomes

- This work has been presented at the Military Health Research Symposium in 2014
- The dosing study will be presented at the Fall American Chemical Society Meeting
- A manuscript for publication is in process based on the findings and, in particular, dealing with the CARPA response. We are completing the analysis of the data collected from the serum samples from all of the swine before manuscript submission, but we anticipate submitting this in November 2015.
- An NIH grant is under review to look at the relationship between particle chemistry and CARPA in October 2015
- Discussions are taking place with Bayer pharmaceuticals about follow up work and the potential for licensing
- A patent has been secured based on engineering particles to avoid CARPA (PCT/US2014/034176)
- The timeline for approaching the FDA is dependent on completing a successful dosing study and performing safety studies with human blood. We are currently looking for support for this work. If we secure funding, we can complete this work in 2-5 years depending on the level of support.
- There are several follow on steps for this work. The first is to optimize the peptide density as we did in our work with the previous formulation. This is critical for translation of the work. The second step we need to address is to do a broader dosing study with the optimized system. We are in the process of submitting grants for funding to continue this work. With this, and safety studies with human blood in vitro, we believe we will be able to scale up production and approach the FDA.

Conclusion

This has been one of the most complicated pieces of science undertaken by this group of researchers. The initial expectation was a focus on making particles in large batches and testing them at different doses to find the optimal dose to stop bleeding in swine.

However, a major portion of the project was spent attempting to determine why the swine were bleeding out, and then working to engineer new particles that would not trigger the problem. Substantial time was spent looking at the CARPA response because swine are not the only species that exhibit CARPA. People do, too, with 10% of the population estimated to have significant CARPA responses (Merkel et al. 2011; Szebeni et al. 2011; Basile et al. 2012; Szebeni et al. 2012; Szebeni et al. 2012; Szebeni et al. 2012).

Uncontrolled bleeding is the leading cause of death in battlefield traumas (Champion et al. 2003) and immediate intervention is one of the most effective means of minimizing mortality associated with severe trauma (Regel e al. 1997). Therefore, these particles must be made safe enough to deploy in the field.

This work has laid the foundation to do so. Two major points could lead to protocols for pursuing higher doses: peptide density optimization, as occurred with the previous formulation and that is critical for translation of the work; and a broader dosing study with the fully optimized system. Through the securing of funds, work could continue along these lines and be completed in 2-5 years. Completion of the dosing study and human blood in vitro studies will enable scale up of production and a subsequent approach of the Food and Drug

While more comprehensive investigation is desired of the nanoparticle features that trigger CARPA, this work has shown that CARPA-free particles can be made and an optimal dose of these particles found to stop bleeding. It is critical work towards moving this technology to patients.

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Meeting Abstracts from this Work

"Synthetic Platelets in Large Animal, Preclinical Models of Trauma-Challenges and Solutions" Military Health Service Research Symposium, August 2013.

We have developed synthetic platelets or intravenously administered hemostatic nanoparticles that reducing bleeding and improve outcomes in a number of models of trauma in rodents including femoral artery injuries, liver injuries, and blast traumas. Based on this, we have begun testing these hemostatic nanoparticles following a blunt trauma liver injury in swine.

The swine liver injury model has been developed to mimic non-compressible injuries sustained by military personnel and permits direct comparison to other hemostatic interventional studies. Briefly, the left lobe of the liver is isolated and hemisected followed by closure of the cavity and quantification of blood loss over time as a function of treatment regime coupled with continuous monitoring and blood analysis.

Initially, we found that even low doses (0.2 mg/kg) led to excessive bleeding. Testing of particles with uninjured swine demonstrated a strong complement-associated response which correlated with the charge on the nanoparticles. We re-engineered the particles to have a neutral charge and saw that this mitigated the complement response.

Intravenous administration of neutrally-charged hemostatic nanoparticles reduced bleeding time in this large animal model of trauma. This work shows that these particles may be an effective means for controlling bleeding in non-compressible injuries and that we must carefully engineer nanotechnologies to deal with potential adverse effects.

"Overcoming CARPA while Stopping Internal Bleeding with Hemostatic Nanoparticles", American Chemical Society Meeting, Fall 2014.

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INTRODUCTION

Traumatic injury is the leading cause of mortality globally, and accounts for 10% of all deaths in the US. Blood loss is the primary cause of death a acute time points post injury in both civilian[1] and battlefield traumas[2]. It has been demonstrated that immediate halting of blood loss is one of the mos effective means of minimizing mortality associated with severe trauma. Currently, there are no effective non-invasive methods to control interna bleeding.

BACKGROUND

Design of I.V. administered hemostatic nanoparticles. Our lab has created novel hemostatic nanoparticles. They are nanoparticles with a poly(lactic co-glycolic) acid (PLGA)- Poly-L-lysine (PLL) nanosphere core about 300nm in size. The nanoparticles are conjugated to polyethylene glyco (PEG) and functionalized with an Arginine- Glycine- Aspartic Acid (RGD) moiety, which cross-links endogenously activated platelets (figure 1).

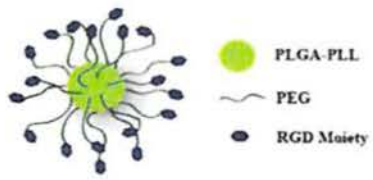

Figure 1. Schematic of Nanoparticle design.

Previous Validation. We have previously demonstrated that our hemostatic nanoparticles can reduce blood loss and fatality in a rat liver injury model. We were able to demonstrate an almost 2 fold increase in survival with the administration of our hemostatic nanoparticles (GRGDS) when compared with saline treatment (Figure 2)[3].

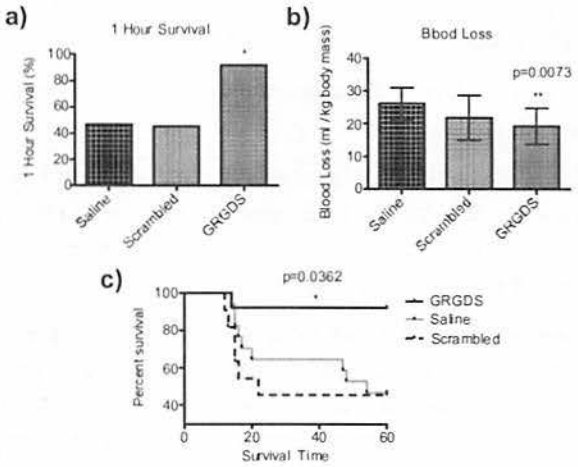

Figure 2. a & c) Survival is significantly increased by treatment with hemostatic GRGDS-functionalized nanoparticles (in part a p=0.040). b) Blood loss is significantly reduced by treatment with hemostatic GRGDS-functionalized nanoparticles

Porcine Liver Injury Model. After validation in the rat liver injury model we moved to a larger animal model, the swine. The swine model was chosen because their cardiovascular system is similar to that of the human cardiovascular system. When changing animal models we optimized our particles by modifying different parameters that would increase hemostatic efficiency, improve dosing, and circumvent undesirable immune responses including complement activation-related pseudo allergy.

COMPLEMENT ACTIVATION-RELATED PSEUDO ALLERGY (CARPA)

CARPA is a reaction to foreign bodies which causes an over activation of the complement system which can lead to symptoms such as tachycardia, bradycardia, arrhythmias, systemic hyper or hypotension, skin flushing and rash, bronchospasm, decreased cardiac output, increased adenosine, and more[4]. The zeta potential of a nanoparticle can be correlated with whether or not it causes CARPA. One of the parameters that we investigated is the RGD moiety that is conjugated to the nanoparticles and its effects on the zeta potential of our nanoparticles (figure 3).

DISCUSSION

We were able to modify a number of different parameters from our original hemostatic nanoparticle including PEGylation, surface charge and size to optimize their performance in a large animal model. Overall our data suggest that our synthetic platelets have the potential to mitigate hemorrhage after traumatic injury.

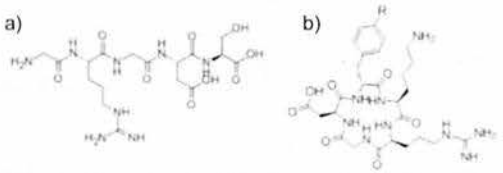

Figure 3. a) Linear GRGDS moiety leads to a highly negative zeta potential when conjugated to our nanoparticles. b) cRGDfk moiety leads to a neutral zeta potential when conjugated to our nanoparticles.

ACKNOWLEDGEMENTS

Support has been provided by NIH Innovator Grant 1DP2OD007338-01, DoD Contract N62645-12-C-4055, NIH grant T32 GM007250, and NIH Grant.

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List of Personnel Receiving Pay from this Work

Rebecca Groynom. Laboratory Research Assistant III. Ms. Groynom was instrumental in all of the surgeries and postmortem analysis of the findings.

Eric Soehnlen, PhD. Eric was a research associate who worked on the project between 9/12 and 1/13. He was responsible for helping with surgeries and making particles.

Jeffrey Ustin, MD. Jeff came in one day per week throughout the study to assist with the surgeries and data analysis. Dr. Ustin is a trauma surgeon who helped us develop the model and played critical roles in assessing the outcomes.